Morphological and molecular barcode analysis of the medicinal tree *Mimusops coriacea* (A.D.C). Miq. collected in Ecuador (#34560)

First revision

Guidance from your Editor

Please submit by **21 Jul 2019** for the benefit of the authors (and your $200 publishing discount).

Structure and Criteria
Please read the 'Structure and Criteria' page for general guidance.

Custom checks
Make sure you include the custom checks shown below, in your review.

Raw data check
Review the raw data. Download from the materials page.

Image check
Check that figures and images have not been inappropriately manipulated.

Privacy reminder: If uploading an annotated PDF, remove identifiable information to remain anonymous.

Files

Download and review all files from the materials page.

1 Tracked changes manuscript(s)
1 Rebuttal letter(s)
7 Figure file(s)
4 Table file(s)
1 Raw data file(s)
1 Other file(s)

Custom checks

DNA data checks

- Have you checked the authors data deposition statement?
- Can you access the deposited data?
- Has the data been deposited correctly?
- Is the deposition information noted in the manuscript?

Structure and Criteria

Structure your review

The review form is divided into 5 sections. Please consider these when composing your review:

1. **BASIC REPORTING**
2. **EXPERIMENTAL DESIGN**
3. **VALIDITY OF THE FINDINGS**
4. General comments
5. Confidential notes to the editor

You can also annotate this PDF and upload it as part of your review

When ready submit online.

Editorial Criteria

Use these criteria points to structure your review. The full detailed editorial criteria is on your guidance page.

BASIC REPORTING

- Clear, unambiguous, professional English language used throughout.
- Intro & background to show context. Literature well referenced & relevant.
- Structure conforms to PeerJ standards, discipline norm, or improved for clarity.
- Figures are relevant, high quality, well labelled & described.
- Raw data supplied (see PeerJ policy).

EXPERIMENTAL DESIGN

- Original primary research within Scope of the journal.
- Research question well defined, relevant & meaningful. It is stated how the research fills an identified knowledge gap.
- Rigorous investigation performed to a high technical & ethical standard.
- Methods described with sufficient detail & information to replicate.

VALIDITY OF THE FINDINGS

- Impact and novelty not assessed. Negative/inconclusive results accepted. *Meaningful* replication encouraged where rationale & benefit to literature is clearly stated.
- All underlying data have been provided; they are robust, statistically sound, & controlled.
- Speculation is welcome, but should be identified as such.
- Conclusions are well stated, linked to original research question & limited to supporting results.

Standout reviewing tips

3

The best reviewers use these techniques

Tip	*Example*
Support criticisms with evidence from the text or from other sources	*Smith et al (J of Methodology, 2005, V3, pp 123) have shown that the analysis you use in Lines 241-250 is not the most appropriate for this situation. Please explain why you used this method.*
Give specific suggestions on how to improve the manuscript	*Your introduction needs more detail. I suggest that you improve the description at lines 57- 86 to provide more justification for your study (specifically, you should expand upon the knowledge gap being filled).*
Comment on language and grammar issues	*The English language should be improved to ensure that an international audience can clearly understand your text. Some examples where the language could be improved include lines 23, 77, 121, 128 – the current phrasing makes comprehension difficult.*
Organize by importance of the issues, and number your points	*1. Your most important issue* *2. The next most important item* *3. …* *4. The least important points*
Please provide constructive criticism, and avoid personal opinions	*I thank you for providing the raw data, however your supplemental files need more descriptive metadata identifiers to be useful to future readers. Although your results are compelling, the data analysis should be improved in the following ways: AA, BB, CC*
Comment on strengths (as well as weaknesses) of the manuscript	*I commend the authors for their extensive data set, compiled over many years of detailed fieldwork. In addition, the manuscript is clearly written in professional, unambiguous language. If there is a weakness, it is in the statistical analysis (as I have noted above) which should be improved upon before Acceptance.*

Morphological and molecular barcode analysis of the medicinal tree *Mimusops coriacea* (A.D.C). Miq. collected in Ecuador

Katherine Bustamante [1], **Efrén Santos-Ordóñez** [Corresp., 2, 3], **Migdalia Miranda** [4], **Ricardo Pacheco** [2], **Yamilet Guitiérrez** [5], **Ramón Scull** [5]

[1] Facultad de Ciencias Químicas. Ciudadela Universitaria "Salvador Allende", Universidad de Guayaquil, Guayaquil, Ecuador
[2] Centro de Investigaciones Biotecnológicas del Ecuador, ESPOL Polytechnic University, Escuela Superior Politécnica del Litoral, ESPOL, Guayaquil, Ecuador
[3] Facultad de Ciencias de la Vida, ESPOL Polytechnic University, Escuela Superior Politécnica del Litoral, ESPOL, Guayaquil, Ecuador
[4] Facultad de Ciencias Naturales y Matemáticas, ESPOL Polytechnic University, Escuela Superior Politécnica del Litoral, ESPOL, Guayaquil, Ecuador
[5] Instituto de Farmacia y Alimentos, Universidad de La Habana, Ciudad Habana., Cuba

Corresponding Author: Efrén Santos-Ordóñez
Email address: gsantos@espol.edu.ec

Background

Mimusops coriacea (A.D.C). Miq., ~~a species from the~~ (Sapotaceae) ~~Family~~, originated from Africa, ~~. *Mimusops coriacea*~~plants were introduced to coastal areas in Ecuador ~~with material from the tree~~where it is now extensively used as a traditional medicine to treat various human diseases ~~in Ecuador~~. Different therapeutically uses of the species include: analgesic, antimicrobial, hypoglycemic, inflammation and pain relieve associated with bone~~s~~ and articulation-related diseases. Furthermore, *M. coriacea* could be used as anti-oxidant agent. However, botanical, chemical, or molecular barcode information related to this much used species is not available from Ecuador. In this study, morphological characterization was performed from ~~in different plant tissues including~~ leaves, stem and seeds. Furthermore, genetic characterization was performed using molecular barcodes for *rbc*L, *mat*k, ITS1 and ITS2 using DNA extracted from ~~-~~leaves.

Methods

Macro-morphological description was performed o~~i~~n fresh ~~plant material including~~ leaves, stem and seeds. For anatomical evaluation, tissues were embedded in paraffin and transversal dissections were done following incubation with sodium hypochlorite and safranin for coloration and fixated later in glycerinated gelatin. DNA extraction was performed using a modified CTAB protocol from leaf tissues, while ~~and~~ amplification by PCR was accomplished for the molecular barcodes *rbc*L, *mat*K, ITS1 and ITS2. Sequence analysis was performed using blast in the GenBank, ~~and p~~Phylogenetic analysis was performed with accessions queried ~~–~~in the GenBank belonging to the subfamily ~~-~~Sapotoideae.

Results

Leaf size was ~~for the length of~~ 13.56 ± 1.46 cm x~~and~~ 7.49 ± 0.65 cm ~~for the width~~; where is macro-morphological description of the stem (see methods), while the fruit is ~~—~~rounded, ~~and~~ containing~~s~~ one or two seeds. The peel of the seeds is dark brown. Sequence analysis revealed that amplicons were generated using the four barcodes selected. Phylogenetic analysis indicated that the barcodes *rbc*L and *mat*K, were not discriminated between species, and [different genus were grouped in one clade of the subfamily Sapotoideae – unclear rephrase]. On the other ~~hand, the ITS1 and ITS2 were discriminative at the~~ level of genus and species of the Sapotoideae.

Manuscript Title

Morphological and molecular barcode analysis of the medicinal tree *Mimusops coriacea* (A.D.C). Miq. collected in Ecuador.

Authors

Katherine Bustamante [1], Efrén Santos-Ordóñez[2, 3], Migdalia Miranda[4], Ricardo Pacheco-Coello[2], Yamilet Gutiérrez[5], Ramón Scull[5].

[1]Universidad de Guayaquil. Facultad de Ciencias Químicas. Ciudadela Universitaria "Salvador Allende". Ave. Kennedy S/N y Av. Delta. Guayaquil. Ecuador telef. 593- 2293680/2293379.

[2]ESPOL Polytechnic University, Escuela Superior Politécnica del Litoral, ESPOL, Centro de Investigaciones Biotecnológicas del Ecuador, Campus Gustavo Galindo, Km. 30.5 vía Perimetral, P.O. Box 09-01-5863, Guayaquil, Ecuador

[3]ESPOL Polytechnic University, Escuela Superior Politécnica del Litoral, ESPOL, Facultad de Ciencias de la Vida, Campus Gustavo Galindo, Km. 30.5 vía Perimetral, P.O. Box 09-01-5863 Guayaquil, Ecuador.

[4]ESPOL Polytechnic University, Escuela Superior Politécnica del Litoral, ESPOL, Facultad de Ciencias Naturales y Matemáticas. Campus Gustavo Galindo. Km 30.5 vía Perimetral. Guayaquil. Ecuador. Email.

[5]Instituto de Farmacia y Alimentos. Universidad de la Habana. 222 y Ave 23. La Coronela. La Lisa. Ciudad Habana. Cuba.

Corresponding Author:

Efrén Santos-Ordóñez[2, 3]

[2]ESPOL Polytechnic University, Escuela Superior Politécnica del Litoral, ESPOL, Centro de Investigaciones Biotecnológicas del Ecuador, Campus Gustavo Galindo, Km. 30.5 vía Perimetral, P.O. Box 09-01-5863, Guayaquil, Ecuador

[3]ESPOL Polytechnic University, Escuela Superior Politécnica del Litoral, ESPOL, Facultad de Ciencias de la Vida, Campus Gustavo Galindo, Km. 30.5 vía Perimetral, P.O. Box 09-01-5863 Guayaquil, Ecuador.
Email address: gsantos@espol.edu.ec

Abstract

Background

Mimusops coriacea (A.D.C). Miq. a species from the Sapotaceae Family, originated from Africa. *Mimusops coriacea* plants were introduced to coastal areas in Ecuador with material from the tree now extensively used as traditional medicine to treat various human diseases in Ecuador. Different therapeutically uses of the species include: analgesic, antimicrobial, hypoglycemic, inflammation and pain relieve associated with bones and articulation-related diseases. Furthermore, *M. coriacea* could be used as anti-oxidant agent. However, botanical, chemical, or molecular barcode information related to this much used species is not available. In this study, morphological characterization was performed in different plant tissues including leaves, stem and seeds. Furthermore, genetic characterization was performed using molecular barcodes for *rbc*L, *mat*k, ITS1 and ITS2 using DNA extracted from leaves.

Methods

Macro-morphological description was performed in fresh plant material including leaves, stem and seeds. For anatomical evaluation, tissues were embedded in paraffin and transversal dissections were done following incubation with sodium hypochlorite and safranin for coloration and fixated later in glycerinated gelatin. DNA extraction was performed using a modified CTAB protocol from leaf tissues and amplification by PCR was accomplished for the molecular barcodes *rbc*L, *mat*K, ITS1 and ITS2. Sequence analysis was performed using blast in the GenBank and phylogenetic analysis was performed with accessions queried in the GenBank belonging to the subfamily Sapotoideae.

Results

Leaf size was for the length of 13.56 ± 1.46 cm and 7.49 ± 0.65 cm for the width; while the fruit is rounded and contains one or two seeds. The peel of the seeds is dark brown. Sequence analysis

revealed that amplicons were generated using the four barcodes selected. Phylogenetic analysis indicated that the barcodes *rbc*L and *mat*K, were not discriminated between species, and different genus were grouped in one clade of the subfamily Sapotoideae. On the other hand, the ITS1 and ITS2 were discriminative at the level of genus and species of the Sapotoideae.

Introduction (are all of the 1st paragraph from just one source, namely Sánchez (2011)?)

In the genus *Mimusops* (Sapotaceae), ~~a total of~~ 45 species have been described that are distributed in Asia, Africa, Australasia and Oceania. In Ecuador there is ? species. Although ~~-~~*Mimusops coriacea* (ADC – in abstract it is indicated as A.D.C.) Miq, has been cultivated widely in the tropics for centuries, it is native only to Madagascar and the Comoro Islands. In Ecuador it has a restricted distribution along the coastal regions of ….

~~72~~73 (focus morphological description only on leaves, stem and seeds –as indicated in the Abstract)

Mimusops spp. are trees reaching a height of up to 25 meters, with ~~—~~a dense cope and an ~~73~~74 irregular short trunk, which exhibit a cracked bark structure. The tree contains simple alternate leaves that

~~74 are alternated and clustered with~~ a brilliant green color ~~green~~. ~~In Ecuador, inventory of *M. coriacea* is 75 lacking; however, a restricted abundance is observed mainly in coastal regions, in areas where the 76 soil is well drained and with a rainy climate. *M. coriacea* trees shared the habitat with other tree 77 species, some of them of the Sapotacea family. Trees from the *M. coriacea*. have been observed 78 in the Amazon has also been reported. Trees from the *M. coriacea* specie are evergreen tree, 79 somewhat tortuous, that can reach up to 20 meters high, with dense and somewhat irregular crown 80 and a short trunk, with very cracked bark. Simple, alternate leaves, usually grouped towards the 81 end of the twigs, with elliptical or obovate to oblong elliptical sheet of up to 20 x 11 cm, base~~ ~~82~~75 ~~obtuse, margin entire and slightly revolute and obtuse or rounded apex are observed.~~ Leaves show ~~83~~76 thick and leathery texture, glabrous, ~~bright green,~~ with the central nerve highlighted and 10-20 ~~84~~ pairs of lateral nerves. ~~Petiole are 1-1.5 cm long, at first pubescent, then glabrous. The axillary 85 inflorescences, are compose of solitary flowers or fascicles of 2-6 flowers, white, aromatic, on 86 hairy pedicels 5-7 (-8) cm long. The calyx contains 8 triangular sepals, 11-12 mm long, with brown 87 hairs externally; while the outer sepals are slightly larger than the interior ones. The corolla with a 88 tube is about 2 mm long and contains 8t lobes of 9-10 mm long, each with 2 deeply lacunar 89 appendages. The androceo contains 8 stamens opposed to the petals, with filaments 3-3.5 mm long, 90 hairy at the base, and the anthers are 4-4.5 mm long, alternating with 8 staminodios of about 5 mm 91 long and hairy on the external side. The conical pubescent ovary, contains 8 locules with glabrous 92 style. The fruit is a sub-spherical berry of 3-4 cm in diameter, on a peduncle more than 6 cm long,~~

~~yellowish at maturity, with a sweet and fleshy floury, edible pulp,~~ Fruits contain~~ing~~ one to several ellipsoid seeds, yellowish brown (Sánchez, 2011).

This species~~, as well as others of the genus,~~ is used for ~~different~~ various medicinal purposes (this paper focus on LEAVES, STEM and SEEDS (see abstract) – thus ethomedicine should also just focus on these 3 plant structures): ~~the cooked~~ ~~bark is used as a painkiller for liver colic and intestinal pain (López et al., 2006);~~ the decoction of the stems is considered useful as a tonic and febrifuge; the tender stems are useful in the treatment of urethroorrhea (Manjeshwar et al., 2011), cystorrhea, diarrhea and dysentery (~~Manjeshwar et al., 2011;~~ Semenya, et al., 2012); and traditional use for the treatment of d~~D~~iabetes is noted (if for diabetes – not linked to either leaves, stem or seeds – then remove). Traditionally in Ecuador, *M. coriacea* is used as an analgesic and anti-inflammatory (Erazo, 2010) (if not linked to either leaves, stem or seeds – then remove). ~~Chivandi et al. (2016) noted the species industrial application. The most important industrial use refers to the latex obtained by shallow incisions in the trunk bark and is used in the preparation of insulating layers for electrical cables, waterproof sheets, plants for waterproof footwear, root canals in dentistry, and the industry of chewing gum and golf balls (Rivera et al., 2013).~~

~~For similar species, including *M. elengi*, triterpenoids have been described (Fayek et al., 2012), phenolics and flavonoids (Chanda et al. 2010a; Baky et al. 2016) and fatty acids (Chivandi et al., 2016), among others.~~ For the genus *Mimusops*, different pharmacological properties have been indicated including antioxidant (~~Rao et al., 2011; Kar et al., 2012~~; Gilliani and Shahwar, ~~–~~2017), anti-inflammatory (Konuku et al., 2017), antimicrobial activities (~~Baliga et al., 2011; Gami, et al., 2012~~; Kiran Kumar et al. 2014) and hypoglycemic activity (Saradha et al. 2017) (if not linked to either leaves, stem or seeds – then remove). ~~In Ecuador, the studies in *Mimusops* are limited to pharmacognostic evaluations of leaves and stems and to the analysis of the oil of the seeds, which have been performed by this research group and is in preparation for publication.~~

Mimusops coriacea is an important medicinal species in Ecuador, however, little is known about the morphological and anatomical characteristics of leaves, stems and seeds; as well as the molecular barcode. Molecular barcodes will be as a complement for proper species identification. Several molecular barcodes have been used in medicinal plants for these purposes (reviewed by Techen et al., 2014); including *rbc*L, *mat*K, ITS1 and ITS2. Although differentiation at the species level is not suitable by using the *rbc*L and *mat*K; the ITS have shown to discriminate at the species level (Techen et al., 2014; Zhang et al., 2015). Furthermore, barcodes

could be used to study patterns of diversifications of the Sapotaceae (Armstrong et al., 2014) and for phylogenetic relationships of different genera (Gautier et al., 2013). This study investigated morphological and molecular barcode characteristics of the *M. coriacea* to will support subsequent

chemical and pharmacological studies, especially for morphological and molecular validation and phylogenetic studies.

.

Materials and Methods

Study area description

Plant material was collected during the month of May 2018 at the "Jardín Botánico", a protected natural vegetative area located in the North zone of "Las Orquídeas" area, next to the Ave. Francisco de Orellana Avenue, in the hills of "Cerro Colorado" of Guayaquil city, Guayas Pprovince, Ecuador (coordinates 02 ° 12'13.6800 "S 079 ° 53'50.6400" W). The area is located –in an altitudinal belt between 50 and 200 m a.s.l., with in a tropical dry forest climate, with alluvial and sedimentary soils, cumulative rainfall of 1150 mm/year, with monthly average temperatures of 31.1º C in winter and 22.6º C in summer, average mean relative humidity of 72% and total evaporation of 1638.7 mm per year (Rosero et al., 2010).

Morphological analysis

Samples were collected from three adult plants identified by a botanist. Furthermore, trees are labeled. Trees with approximately 30 m in height, with flowers and fruits were selected forvia random sampling randomly. One branch containing leaves, fruits and flowers is placed at the GUAY herbarium of Guayaquil University, where the botanists analyzed the samples with taxonomic characters, following proper classification and assignation of a number. Samples from the *M. coriacea* was assigned the accession number 13111.

Morphological description of different organs was performed on fresh and mature leaves (n=100), – stems and seeds with a stereoscope (model: Zeizz LUMAR.V12, adapted with an ACXION MRc5 camera. AXION VISION Rel 4.8 (Zeizz, Germany) software was used in, accordance to the method of (Miranda and Cuéllar (2000) to analyze leaf (n=100) shape, edge, apex, base, petiole, venation, consistency, and color. Size -was measured in micrometer. For the stems, —the characteristics analyzed includes shape, color, external and internal surfaces, and fracture. For fruit characterization, 60 fruits and extracted seeds were analyzed in shape and dimensions, seed coat, and endosperm.

Formatted: Font: Italic

Formatted: Font: Italic

For histological analysis, transversals cuts of fresh leaves were performed manually, which were hydrated and clarified with 1% sodium hypochlorite. Tissues were colored with 1% safranin in water, following fixation with glycerinated gelatin, according to Gattuso and Gattuso (1999). To analyze anatomical aspects of the leaf epidermis, a longitudinal cut followed with a diaphanization technique was performed. Cleared leaves were obtained with sodium hypochlorite following incubation with 1% safranin in water. Micro-morphological characteristics of cortex were performed to the drug in powder, performing histochemical reactions including: starch determination (Lugol reagent), lignine (1% saphranine in water), and essential oil (5% Sudan III solution in 70% ethanol) (Gattuso and Gattuso, 1999). Micromorphology of seeds was performed using dried fragmented material following the procedure described above for leaves and cortex.

DNA extraction and PCR

Leaves from collected samples from one specimen were ground using liquid nitrogen in the grinder MM400 (Retsch) and stored at -80°C upon DNA extraction. Approximately, 100 mg of leaf was used for DNA extraction using a CTAB protocol with some modifications (Pacheco Coello et al. 2017). PCR was performed using the 2x GoTaq® master mix (Cat. # M7123, Promega) using 0.5 µM of each primer (Table 1). The final volume was 50 µl per reaction. PCR conditions were 95°C to start denaturation; 35 cycles of: 95°C for 30 s, 60°C (for *rbc*L) or 56°C (for *mat*K, ITS1 and ITS2) for 30 s, 72°C for 90 s, with a final extension of y 72°C for 5 min. Five microliter of PCR reaction was loaded on a 1.5% gel to check for the presence of amplicons. The remaining 45 µl were purified using the Wizard SV Gel and PCR Clean-Up System (Cat. # A9282, Promega) and sequenced commercially (Macrogen, Maryland, USA). At least three technical replicates were sequenced and a consensus was developed.

Bio-informatics analysis of sequences

Sequences were trimmed from low quality using FinchTV or Chroma's 2.6.5 (Technelysium). Processed sequences were blast (Zhang et al. 2000) in the GenBank using the nucleotide database. Sequences from the Subfamily Sapotoideae were selected (GenBank) for phylogenetic analysis using MEGA 7.0.26 (Kumar et al., 2016) including *Mimusops caffra* (HF5422847), *Mimusops*

elengi (KF686246), *Palaquium amboinense* (HF542854), among others. For each barcode, the recommended model from the MEGA7 was used for the phylogenetic analysis after alignment with MUSCLE. For the phylogenetic analysis, the Maximum Likelihood methods was used for each barcode using bootstrap test (100 replicates).

Results

Morphological evaluation of the leaves

The ~~macro-morphological evaluation allowed the observation~~leaves ~~of~~ were oblong ~~leaves of~~with a coriaceous-waxy texture, containing a short petiole, retuse apex, entire border and an obtuse base. Macroscopic details of the leaves are ~~shown~~ illustrated in ~~(~~Figure~~.~~ 1~~)~~. In respect to the dimensions of the leaves (n=100), the average value observed for the length of the leaves was 13.56 ± 1.46 cm and 7.49 ± 0.65 cm for the width.

Morphological evaluation of the crust~~:~~

The crust presented a rugose cuticle ~~of~~ with an intense gray color, ~~with~~ and a ~~n underneath~~ slightly brown outer abaxial surface (Fig. 2A) with rough streaks. The internal surface was reddish ~~-~~brown, fibrous and furrowed (Fig. 2B).

Morphological evaluation of the seeds~~:~~

In the macro-morphological study, the length and width of the green and ripe fruits (n=?), the seeds (n=?) with the husk and the endosperm of the seeds were considered (Fig. 3). The fruit is rounded and contains one or two seeds. The seeds with a peel are dark brown. The dimensions are presented in ~~(~~Table 2~~)~~. 209

Anatomical evaluation~~:~~

Leaves: In the leaf anatomy at the level of a cross section of the central nerve (Fig. 4A), the adaxial surface is convex, slightly wavy ~~and~~ with the abaxial face ~~is~~ concave. An enlarged view of the ~~-~~nerve (Fig. 4B) shows a cuticle of waxy texture that covers the entire leaf, and well visible in the macro-morphological study, followed by the epidermis, which is made up of tabular cells, which gives way to ~~the~~ a set of cells that form the spongy parenchyma, given the intercellular spaces which are defined. Possible crystals of calcium oxalate are also observed.

Bordering the central part of the central nerve, there is a cord (Fig. 4C), colored red, corresponding to the endodermis, the structure that surrounds the pericycle. In the middle the conductive tissue formed by the vascular system xylem and phloem is observed (Fig. 4C).

An image of the leaf mesophyll (Fig.4D) shows a somewhat thick cuticle on the abaxial surface, followed by the epidermis, a parenchyma palisade with elongated cells that at times become stratified. In the same way, the entire center of the structure occupied by the spongy parenchyma is observed, which borders on the upper epidermis that ends with the cuticle, previously mentioned.

The diafanization of a portion of the leaf by the adaxial side showed an epidermis with cells of variable shape and size (Fig. 5A). However, the abaxial epidermis ~~evidenced~~ contain a large number of anomocitic type stomata, where the epidermal cells surrounding the pair of occlusive cells are not morphologically different from the rest of the epidermal cells (Fig. 5B). A stain with Sudan III reagent at the level of the epidermis, allowed the visualization of bags with essential oils, which took a reddish coloration (Fig. 5C).

The microscopic analysis of the powder drug showed different fibers and vascular bundles, in this case belonging to the xylematic tissue, classified as scalariform. Figure 5 shows the observed microscopic characteristics.

Bark: The micro-morphological analysis of the powder drug showed different fibers and the vascular system, belonging to the xylematic tissue~~, responsible for the transport of the crude sap to the photosynthetic centers and the circulation of the highest percentage of water.~~ The xylematic vessels are classified as scalariform (Fig. 6).

Seeds: The micro-morphological analysis of the seed powder (Fig. 6), allowed the visualization of a section of the episperm (outer layer of the seed or testa) where the presence of cells of the sclerenchyma tissue corresponding to the supporting tissue is observed. This cell has a well-defined compact arrangement and the walls are slightly thick. The sclerides of the macro-sclerosis type and elements of the conductive tissue was observed. Histochemical reactions on the samples, demonstrated a well-defined red-colored oil pocket that could be observed through the reaction with the Sudan III reagent. Starch granules of ovoid shape and blackish color were ~~also~~ observed when using the Lugol reagent.

Molecular barcode of *M. coriacea*~~.~~

As a complement analysis for characterization and identification of the *M. coriacea* sample, PCR of the molecular barcodes *rbc*L, *mat*K, ITS1 and ITS2 was performed. Amplicons were detected for all the molecular barcodes (Fig. 7). Sequences will be submitted in the GenBank (Table 3).

After alignment of the barcode's sequences from the GenBank with the *M. coriacea* sample, the best model for phylogenetic analysis are shown (Table 4). The phylogenetic analysis revealed that for the barcodes *rbc*L and *mat*K, most of the *Mimusops* spp. are clustered together with other *genera* (Supplementary Figure). On the other hand, the ITS1 and ITS2 sequences revealed several clades for the different genera including the *Mimusops* (Supplementary Figure).

Discussion

Morphological evaluation of the leaves:

The morphological characteristics of the leaves correspond to that reported by Miranda and Cuéllar (2000) and Gami, et al., (2012) for which species?. The venation is a closed type, which corresponds to a reticular system (the veins branch and anastomose with each other forming a network that facilitates the diffusion of liquids); which is very common in the dicotyledons. In this case, of the penninervia type, the vascular system is one of the most advanced systems that ensures nutrition to all parts of the leaf (Gami et al., 2012).

The information referenced in the literature regarding the characteristics of the leaves is scarce limited; thus, comparison with respect to two species of the genus was performed. For *Mimusops elengi* L., Gami et al., (2012) reported that the leaves are elliptical in shape, little slightly acuminate at the apex, glabrous with an acute base, and petioles 1.3 - 2.5 cm in length. The dimensions of the leafve range between 6.3-10.0 cm by 3.2 - 5.0 cm wide, while *Mimusops hexendra* Roxb (without Manilkara *hexendra* Roxb) present oblong leaves, rounded at the apex, glabrous, dark green in the beam and clear on the abaxialunder side, with a dimension of 2.5 – 11 cm long and 1.0 6.0 cm wide (Chanda et al., 2010b). Some species genetically similar to the species under study, present some differences especially in the dimension of the leafve with respect to those study, which are superior.

Morphological evaluation of the crust:

Related to the crust, no referenced information was found.

Morphological evaluation of the seeds:

For the seeds, significant differences were observed between the evaluated parameters of the whole fruits and their seeds at maturity (Gopalkrishna and Shimpi, 2011); for *M. elengi* seed husk was light brown to blackish, with measures of 1.7 -1.9 cm long and 1.2 -1.5 cm wide, with differs from those obtained for the species studied. The endosperm presented dimensions of 1.42 x 1.0 cm when it came from green fruits and 1.43 x 0o.91 cm when it came from ripe fruits, decreasing its thickness in this case

Formatted: Indent: Left: 0.24 cm

Formatted: Font: Italic

288

289 Anatomical evaluation:

Leaves: ~~The microscopic analysis of the powder drug showed different fibers and vascular bundles, in this case belonging to the xylematic tissue, classified as scalariform.~~RESULTS!!

Crust and seeds: Related to the crust and seeds, no referenced information was found ~~para~~ for anatomical characteristics.

Molecular barcode

Analysis of the molecular barcodes is a complement study for the characterization of the *Mimusops* spp. for medicinal application. Molecular barcode is useful for genotyping organisms, and different *loci* have been proposed characterized land plants (CBOL. Plant Working Group, 2009). Although, the two proposed *loci* for barcodes are from plastid genome and includes the *rbcL* and *matK* (Techen et al., 2014), other *loci* including ITS1 and ITS2 are widely used for medicinal plants (Kim et al., 2016). Furthermore, the ITS2 region is suggested as a barcode for species identification over *rbcL* and *matk* (Zhang et al., 2016). Therefore, the phylogenic analysis for differentiation between genera and species is not practical while using *rbc*L and *mat*K. On the other hand, the ITS1 and ITS2 of the present study were in the same clade as the *M. coriacea*. from Madagascar, while the *M. elengi* (accessions KF686246, KF686245, HF542849, KF686245) were in different clades (Supplementary Figure). Furthermore, other molecular barcodes could be included in future analysis by sampling in different regions in Ecuador; and also by comparing with other results of individual specimens from the family Sapotaceae. Other barcodes may include the plastids *rpl32-trnL*, *rps16-trnK*, and *trnS-trnFM* (Armstrong et al., 2014); and *trnH-psbA* spacer, the *trnC-trnD* region (consisting of the *trnC-petN* spacer, the *petN* gene, the *petN-psbM* spacer, the *psbM*gene and the *psbM-trnD* spacer), the *trnC-psbM* region, and the 3′ end of *ndhF* (Richardson et al., 2014). However, the ITS is more variable than the plastids barcodes (Richardson et al., 2014). Further analysis could be performed to evaluate intraspecific and intraspecific variations of different barcodes to even evaluate at subspecies level.

Conclusions

For the first time, the macro and micro-morphological characteristics of the leaves, stems and seeds, of the *M. coriacea* collected in Ecuador were performed. The evaluation of the identity of the species, which is classified taxonomically as *Mimusops* sp., which is a novelty of this work, was confirmed by using molecular barcodes. Most important, the ITS1 and ITS2 indicate more resolution at the species level (*M. coriacea*) than the *rbc*L and *mat*K, confirming published results in medicinal plants. However, further molecular barcode characterization should be performed in *Mimusops* spp. to further validate resolution at the species level as a complement for proper identification using morphological characteristics. Further pharmacognostic analysis will be performed to study medicinal properties of *M. coriacea*.

Acknowledgements

Identification of samples by the GUAY herbarium of the Faculty of Natural Sciences of the Guayaquil University is acknowledged. The study was performed in the framework of the project "*Productos Naturales de interés Agrícola y para la Salud*" from ESPOL University

References **(format not consistently applied)**

Armstrong KE, Stone GN, Nicholls JA, Valderrama E, Anderberg AA, Smedmark J, Gautier L, Naciri Y, Milne R, Richardson JE. 2014. Patterns of diversification amongst tropical regions compared: a case study in Sapotaceae. *Frontiers in Genetics* 5: -362.

Baky MH, Kamal AM, Elgindi MR, Haggag EG. 2016. A Review on Phenolic Compounds from Family Sapotaceae. *Journal of Pharmacognosy and Phytochemistry* 5(2):280–287.

Baliga MS, Pai RJ, Bhat HP, Palatty PL, Boloor R. 2011. Chemistry and medicinal properties of the Bakul (*Mimusops elengi* Linn): a review. *Food Research International*. 44:1823–1829.

CBOL Plant Working Group. 2009. A DNA barcode for land plants. PNAS 106(31):12794–12797.

Chanda S, Nagani K, Parekh J. 2010b. Assessment of Quality of *Manilkara hHexandra* (Roxb.) Dubard Leaf (Sapotaceae): Pharmacognostical and Physicochemical Profile. *Pharmacognosy Journal*. 2(13):520–524. DOI:10.1016/S0975-3575(10)80054-9

Formatted: Font: Italic

Formatted: Font: Italic

Formatted: Font: Italic

Formatted: Font: Italic

Formatted: Font: Italic

~~Chanda SV, Nagani KV. 2010a. Antioxidant Capacity of Manilkara zapota L . Leaves Extracts Evaluated by Four in Vitro Methods. *Journal of Biological Sciences* 8(10): 260–266. DOI:10.7537/marsnsj081010.21~~

Chivandi E, Mukonowenzou N, Berliner D. 2016. The Coastal Red-Milkwood (Mimusops caffra) Seed: Proximate, Mineral, Amino Acid and Fatty Acid Composition. *South African Journal of Botany* 102: 137–141 DOI:10.1016/j.jep.2012.03.008

Erazo N. 2010. Compendio de plantas medicinales del Ecuador. Escuela Superior Politécnica de Chimborazo. Riobamba. Ecuador

Fayek NM, Monem AR, Mossa MY, Meselhy MR, Shazly AH. 2012. Chemical and Biological Study of Manilkara Zapota (L.) Van Royen Leaves (Sapotaceae) Cultivated in Egypt. *Pharmacognosy Research* 4 (2):85-91. DOI: 10.4103/0974-8490.94723.

Gami B, Pathak S, Parabia M. 2012. Ethnobotanical, Phytochemical and Pharmacological Review of Mimusops Elengi Linn. *Asian Pacific Journal of Tropical Biomedicine* 2(9):743–48 DOI:10.1016/S2221-1691(12)60221-4.

Gattuso MA, Gattuso SJ. 1999. Manual de procedimientos para el análisis de drogas en polvo. Editorial de la Universidad Nacional de Rosario Urquiza. Argentina.

Gautier L, Naciri Y, Anderberg AA, Smedmark JEE, Randrianaivo R, Swenson U. (2013) A new species, genus and tribe of Sapotaceae, endemic to Madagascar. Taxon 62(5):972-983

Gillani SS, Shahwar D. 2017. Investigation of Antioxidant Activity in *Mimusops elengi*. J Plant Biochem Physiol 5:202. DOI:10.4172/2329-9029.1000202.

Gopalkrishnan B, Shimpi SN. 2011. Seeds of Mimusops elengi Linn. Pharmacognosy and Phytochemical Studies. *International Journal of Pharmacognosy and Phytochemical Research*. 3(1):13–17.

Kar B, Kumar RBS, Karmakar I, Dola N, Bala A, Mazumder UK, Hadar PK. 2012. Antioxidant and in Vitro Anti-Inflammatory Activities of Mimusops elengi Leaves. *Asian Pacific Journal of Tropical Biomedicine* (2 SUPPL.): S976–80. DOI:10.1016/S2221-1691(12)60346-3.

376 Kim WJ, Ji Y, Choi G, Kang YM, Yang S, Moon BC. 2016. Molecular identification and
377 phylogenetic analysis of important medicinal plant species in genus *Paeonia* based on
378 rDNA-ITS, *mat*K, and *rbc*L DNA barcode sequences. Genetics and Molecular Research
379 15(3). DOI: 10.4238/gmr.15038472gmr.15038472.

380 Kiran Kumar HA, Mandal BK, Mohan Kumar K, Maddinedi Sb, Sai Kumar T, Madhiyazhagan P,
381 Ghosh AR. 2014. Antimicrobial and Antioxidant Activities of Mimusops elengi Seed
382 Extract Mediated Isotropic Silver Nanoparticles. *Spectrochimica Acta - Part A: Molecular*
383 *and Biomolecular Spectroscopy* 130:13–18. DOI:10.1016/j.saa.2014.03.024.

384 Konuku, K., Krishna Ch., Velliyur K., Zenebe H., Haftom K., Tentu KN., Ponce P, Dogulas J.,
385 and Duddukuri G. 2017. "Anti-inflammatory activity of Manilkara zapota leaf extract"
386 International Journal of Current Pharmaceutical Reshaer. 9(4). ISBN-0975-7066.

387 Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis
388 version 7.0 for bigger datasets. Molecular Biology and Evolution 33:1870-1874.

389 López Camacho, René Navarro López, Jaime Alberto Montero González, Martín Iván Amaya
390 Vecht, Karen Rodríguez Castañeda, Misael Polania Barboza, Abraham. René López
391 Camacho. 2006. Manual de identificación de especies no maderables del Corregimiento de
392 Tarapacá, Colombia. Available at: https://www.sinchi.org.co/manual-deidentificacion-de-
393 especies-no-maderables-del-corregimiento-detarapaca. Accessed: 17mayo/2018.

394 Manjeshwar SB, Ramakrishna JP, Harshith PB, Princy LP, Rekha B. 2011. Chemistry and
395 medicinal properties of the Bakul (Mimusops elengi Linn): A review. Food Res Int; 44(7):
396 1823-1829.

397 Miranda MM, Cuéllar AC 2000. Manual de prácticas de laboratorio. Farmacognosia y productos
398 naturales. Ciudad Habana 25-49, 74-79.

399 Pacheco Coello R., Pestana Justo J., Factos Mendoza A., Santos Ordoñez E. 2017. Comparison of
400 three DNA extraction methods for the detection and quantification of GMO in Ecuadorian
401 manufactured food. BMC Research Notes 10:758 DOI:10.1186/s13104-017-3083-x.

402 Rao S., Rani P., Kumar R., and Keshar N. 2011. "Evaluation of in Vitro Antioxidant Activity and
403 Total Phenolic Content of Methanol Bark Extract of Mimusops elengi." Free Radicals and
404 62 Antioxidants 1 (2). Elsevier Masson SAS: 62–71. doi:10.5530/ax.2011.2.11.

405 Richardson JE, Bakar AM, Tosh J, Armstrong K, Smedmark J, Anderberg AA, Slik F, Wilkie P.
406 2014) The influence of tectonics, sea-level changes and dispersal on migration and
407 diversification of Isonandreae (Sapotaceae), *Botanical Journal of the Linnean Society*
408 174(1):130–140.

409 Rivera L. Peñuela, M. Jimenez, E. Vargas, M. 2013. "Ecology and Silviculture de Especies Útiles
410 Amazónicas". Available at: http://www.bdigital.unal.edu.co/36632/6/9789587616347.pdf.
411 Accessed: 17 November 2018.

412 Rocero C., Iturralde G., Zambrano R., Vallardo V. 2010. Ampliación del área nacional de
413 recreación Los Samanes. Ministerio de Ambiente. Ecuador. Available at:
414 imce.ambiente.gob.ec/sites/default/files/documentos/anny/Informe%20ampliación%20Sa
415 manes.pdf. (accessed 15 may 2019)

416 Sánchez JM. 2011. Flora ornamental Española, España. Editorial Mundiprensa. 3-667

417 Saradha S, Ruckmani A, Chokkalingam M, Maignanakumar R, Arunkumar R, Madhavi E,
418 Lakshmipathy Prabhu R. 2014. Hypoglycemic activity of aqueous and ethanolic extracts
419 of Manilkara zapota seeds in streptozotocin induced diabetic rats. Int J Pharm Pharm Sci
420 6(2):434-437

421 Semenya S, Potgieter M, Erasmus L. 2012. Ethnobotanical Survey of Medicinal Plants Used by
422 Bapedi Healers to Treat Diabetes Mellitus in the Limpopo Province, South Africa. *Journal*
423 *of Ethnopharmacology* 141(1):440–45. DOI: 10.1016/j.jep.2012.03.008

424 Techen N, Parveen I, Pan Z, Khan IA. 2014. DNA barcoding of medicinal plant material for
425 identification. Curr. Opin. Biotechnol. 25:103–110.

426 Technelysium. Available at https://www.technelysium.com.au (accessed 2 October 2018)

427 Zhang D, Jiang B, Duan L, Zhou N. 2016. Internal transcribed spacer (ITS), an ideal DNA barcode
428 for species discrimination in Crawfurdia Wall. (Gentianaceae). *African journal of*
429 *traditional, complementary, and alternative medicines* *13*(6):101-106.
430 DOI:10.21010/ajtcam.v13i6.15

431 Zhang Z, Schwartz S, Wagner L, Miller W. 2000, A greedy algorithm for aligning DNA sequences.
432 J ComputBiol 7(1-2):203-14. Available at https://blast.ncbi.nlm.nih.gov/Blast.cgi

433

434 **Figure 1. Macro morphological details of leaf from *M. coriacea.***

435 **A:** retuse apex, **B:** whole edge, **C:** obtuse base, **D**, **E** and **F**: closed rib
436
437 **Figure 2. Macro morphological details of crust from *M. coriacea.***
438 **A**: external surface, **B**: internal surface
439
440 **Figure 3. Macro morphological characters of fruits and seeds from *M. coriacea.***
441 **A:** green fruit, **B:** ripe fruit, **C:** seeds green fruits with peel, **D:** seeds ripe fruits with peel,
442 **E:** endosperm green seeds, **F:** endosperm mature seeds
443
444 **Figure 4. Microscopic characteristics of leaf from *M. coriacea.***
445 **Transversal section of the central nerve of the leaf (I): A:** central nerve of the leaf, **B** and **C:**
446 enlarged view of the central nerve, **D:** mesophilic, Cu: cuticle, Ep: epidermis, COC: calcium
447 oxalate crystals, SP: spongy parenchyma, VS: vascular system, En: endodermis, AdE: adaxial
448 epidermis, PP: palisadeparenchyma, AbE: abaxial epidermis.
449
450 **Figure 5. Microscopic characteristics of leaf from *M. coriacea.***
451 **Diafanized of the leaf (II): A:** adaxial epidermis, **B** and **C:** abaxial epidermis
452 EpC: epidermal cells, S: stomata, EO: essential oils
453
454 **Figure 6. Powder drug characteristics of *M. coriacea.* A:** powder drug from leaf. **B, C, D, E:**
455 powder drug from bark. **F, G, H, I, J:** powder drug from seed.
456 VS: vascular system, F: fibers, S: starch, ST: suberoustissue, SF: septate fibers,
457 COC: calcium oxalate crystal, SC: sclerides cells, MS: macrosclerides, OB: oilbag,
458 SG: starch granules
459
460 **Figure 7. Gel electrophoresis of amplicons generated for the molecular barcodes with the**
461 **genomic DNA of *M.coriacea*.** (A) Amplification of rbcL (rbcLA_F/ rbcLA_R), matK
462 (matK_3F_KIM f/matK_1R_KIM R). (B) Amplification of ITS1 (5a_F/ITS 4_R), and ITS2
463 (S2f/S3R). Numbers from 1 to 3 are technical replicates of DNA of each species. + is the
464 positive control. – is the negative control. M is the 100 bp DNA Ladder (Cat. # G2101,
465 Promega).

466
467
468
469

Table 1(on next page)

Primers used for amplification of *rbc*L, *mat*K, ITS1 and ITS2.

1 Table 1. Primers used for amplification of *rbc*L, *mat*K, ITS1 and ITS2.

Primer pairs	**Sequence**	**Estimated size (bp)**	***Locus***	**Reference**
rbcLA_F/	ATGTCACCACAAACAGAGACTAAAGC	550	*rbc*L	Costion et al. 2011
rbcLA_R	GTAAAATCAAGTCCACCRCG			
matK_3F_KIM	CGTACAGTACTTTTGTGTTTACGAG	850	*mat*K	Costion et al., 2011
f/matK_1R_KIMR	ACCCAGTCCATCTGGAAATCTTGGTTC			
ITS 5a F/	CCTTATCATTTAGAGGAAGGAG	700	ITS1	Schultz et al. 2005
ITS 4 R	TCCTCCGCTTATTGATATGC			
S2F/	ATGCGATACTTGGTGTGAAT	400	ITS2	Schultz et al. 2005
S3R	GACGCTTCTCCAGACTACAAT			

2
3

Table 2(on next page)

Dimensions of the fruits and seeds of *M. coriacea*

1 Table 2. Dimensions of the fruits and seeds of *M.coriacea*

Type of fruit or seed	Length cm	Width cm
Green Fruit	2.97 ± 0.18	3.14 ± 0.25
Ripe Fruit	2.89 ± 0.2	2, 97 ± 0.25
Green Seeds	1.66 ± 0.13	1.15 ± 0.21
Ripe Seeds	1.79 ± 0.09	1.20 ± 0.09

2

Table 3(on next page)

Samples and sequences submitted in the GenBank from the samples of *M. coriacea* barcoded.

1 Table 3. Samples and sequences submitted in the GenBank from the samples of *M. coriacea*
2 barcoded.

Barcode	Accession
*rbc*L	2198607
*mat*K	2199742
ITS1	MK577640
ITS2	MK577643

3

Table 4(on next page)

Best model to describe the substitution pattern using Mega7.

1 Table 4. Best model to describe the substitution pattern using Mega7.

Barcode	Best model
*rbc*L	JC
*mat*K	T92
ITS1	T92+G
ITS2	T92+G

2 KG: Kimura 2-parameter; +G: Gamma distribution; T92: Tamura 3-parameter; GTR: General
3 Time Reversible. K2: Kimura 2-parameter. JC: Jukes-Cantor.
4

Figure 1

Macro morphological details of leaf from *M. coriacea*

A: retuse apex, **B:** whole edge, **C:** obtuse base, **D**, **E** and **F:** closed rib

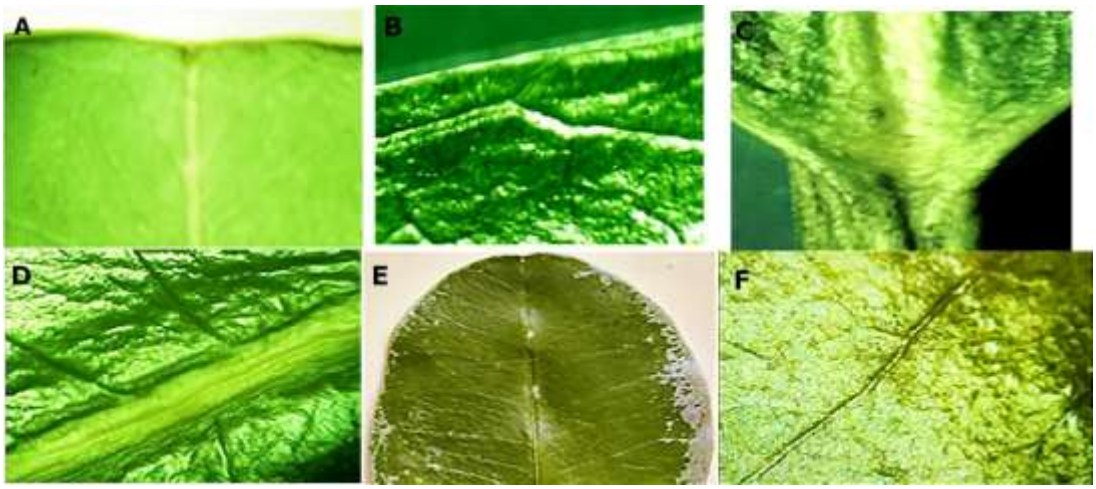


Figure 2

Macro morphological details of crust from *M. coriacea*

A: external surface, **B**: internal surface

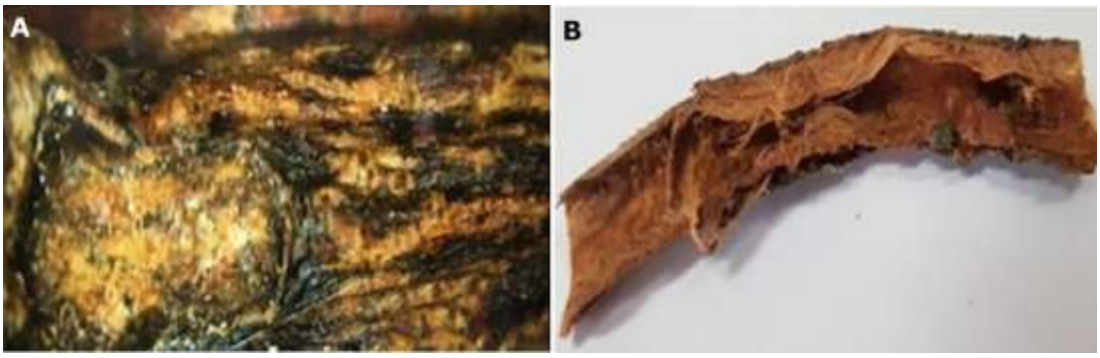


Figure 3

Macro morphological characters of fruits and seeds from *M. coriacea*

A: green fruit, **B:** ripe fruit, **C:** seeds green fruits with peel, **D:** seeds ripe fruits with peel, **E:** endosperm green seeds, **F:** endosperm mature seeds

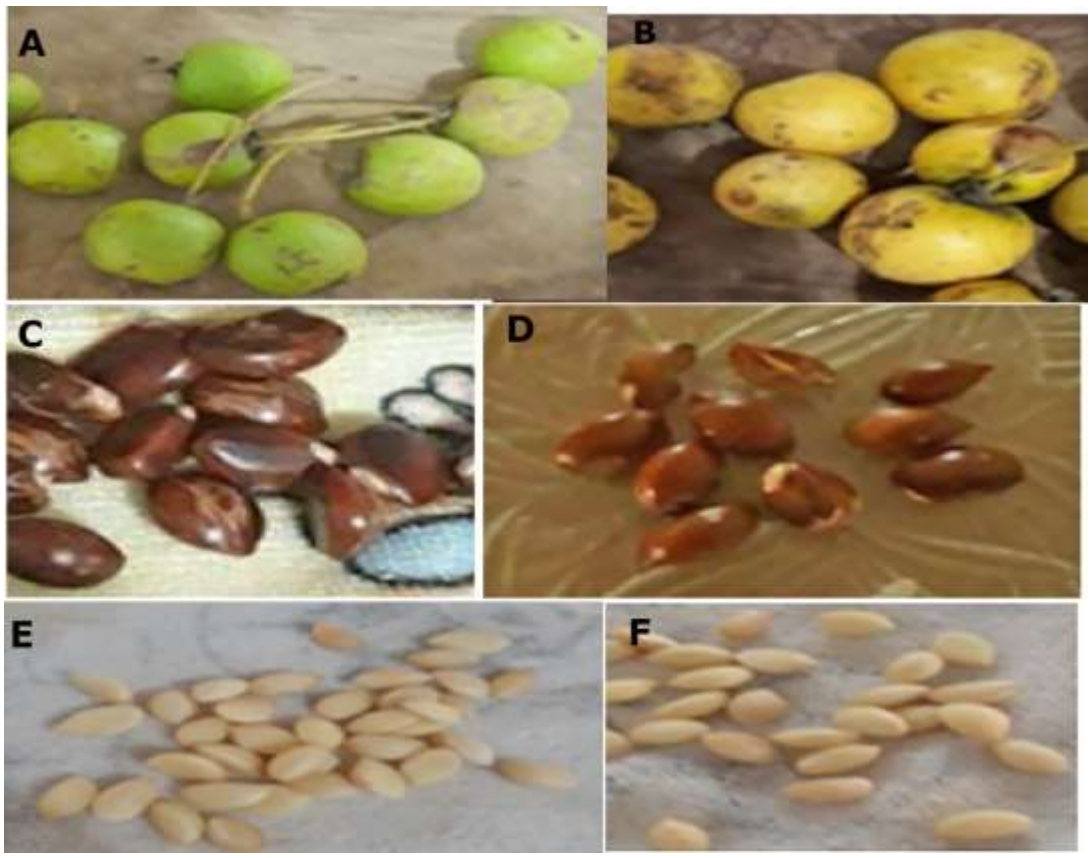


Figure 4

Microscopic characteristics of leaf from *M. coriacea*

Transversal section of the central nerveof the leaf (I): A: central nerve of the leaf, **B** and **C:** enlarged view of the central nerve, **D:** mesophilic, Cu: cuticle, Ep: epidermis, COC: calcium oxalate crystals, SP: spongy parenchyma, VS: vascular system, En: endodermis, AdE: adaxial epidermis, PP: palisadeparenchyma, AbE: abaxial epidermis

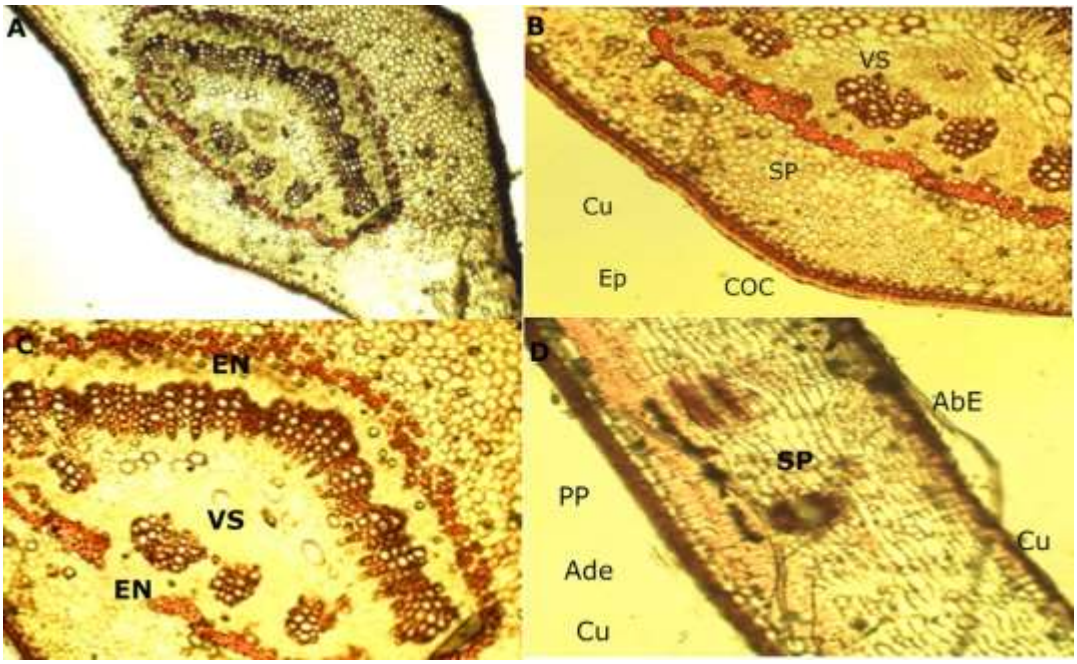


Figure 5

Microscopic characteristics of leaf from *M. coriacea*

Diafanized of the leaf (II): A: adaxial epidermis, **B** and **C:** abaxial epidermis EpC: epidermal cells, S: stomata, EO: essential oils

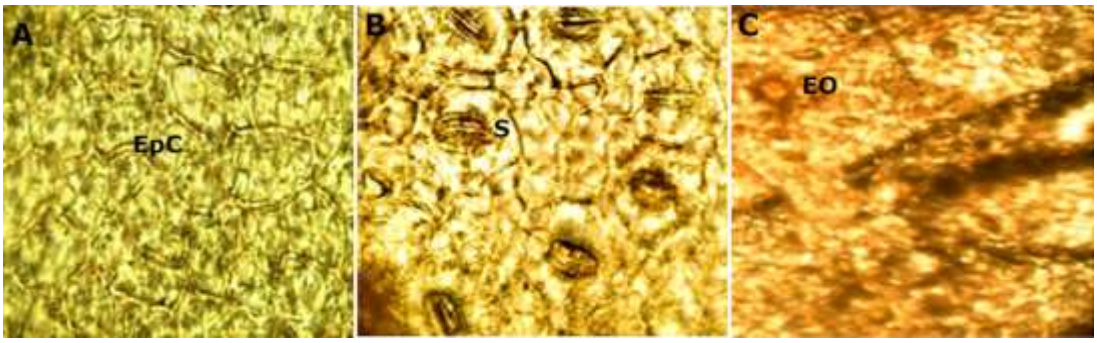


Figure 6

Powder drug characteristics of *M. coriacea*

A: powder drug from leaf. **B, C, D, E:** powder drug from bark. **F, G, H, I, J:** powder drug from seed.VS: vascular system, F: fibers, S: starch, ST: suberoustissue, SF: septate fibers, COC: calcium oxalate crystal, SC: sclerides cells, MS: macrosclerides, OB: oilbag, SG: starch granules

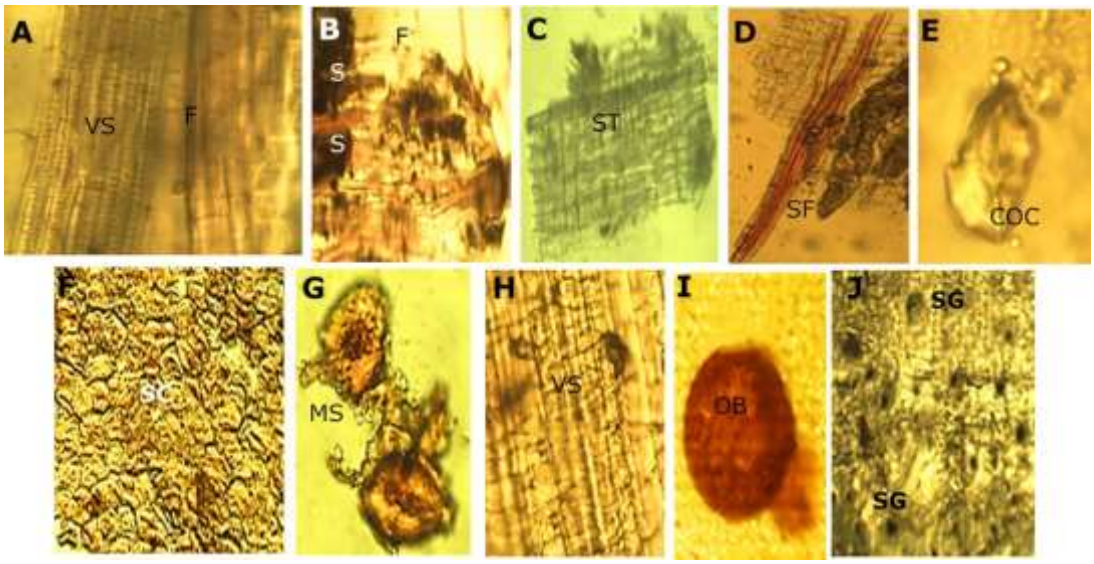
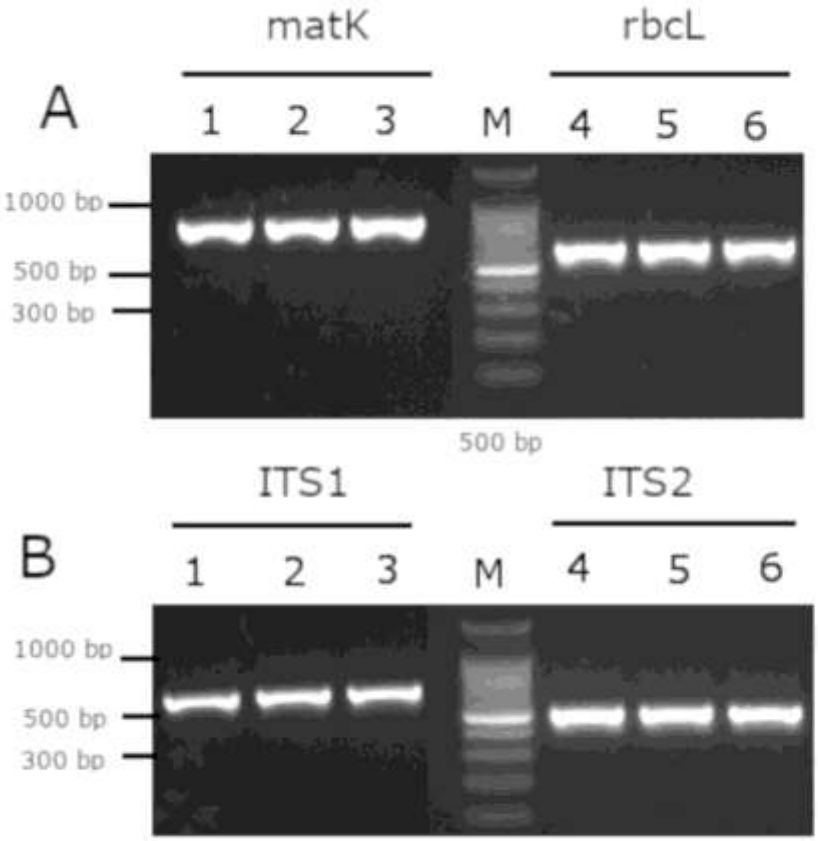


Figure 7

Gel electrophoresis of amplicons generated for the molecular barcodes with the genomic DNA of *M. coriacea*

(A) Amplification of rbcL (rbcLA_F/ rbcLA_R), mat K (matK_3F_KIM f/matK_1R_KIM R). (B) Amplification of ITS1 (5a_F/ITS 4_R), and ITS2 (S2f/S3R). Numbers from 1 to 3 are technical replicates of DNA of each species. + is the positive control. – is the negative control. M is the 100 bp DNA Ladder (Cat. # G2101, Promega).


matK
rbcL
A
1
2
3
M
4
5
6
1000 bp
500 bp
300 bp
500 bp
ITS1
ITS2
B
1
2
3
M
4
5
6
1000 bp
500 bp
300 bp